REMARKS

Claims 7-13 and 20 are pending. Claims 7 and 20 stand rejected as allegedly indefinite; and claims 7-13 were rejected as non-enabled. Claims 7-13 and 20 also were rejected over cited references based on assertions that their subject matter was either anticipated or obvious. Claims 7-12 and 20 are currently amended, and new claims 21-22 have been added.

Claim 7 was amended to make explicit the step of recording the intracellular localization pattern of signal transduction proteins of interest in the absence of any added toxic compound. Since this step would be understood to be necessary by one of ordinary skill, making it explicit adds no new matter. The claim also makes explicit the step of providing a set of toxic compounds; that step is inherent in the original claims, and examples of such toxic compounds are provided in the specification at pg.10, ll. 25 to pg. 11, 7.

In addition, claim 7 now recites that the localization "pattern" comprises information about the amount of the signal transduction protein "concurrently" present "in at least two cellular locations selected from the group consisting of nuclear, perinuclear, diffuse cytoplasmic, cytoplasmic fibril-associated, and membrane-associated locations." The term "localization pattern" is supported throughout the specification, and specifically at page 5, lines 20-21. The limitation "concurrently" is supported by the specification at page 4, line 19, which points out that the methods of detection used permit locations of proteins within a cell to be determined "essentially simultaneously" for creating a profile or 'footprint'. The list of cellular locations where the proteins may be observed in the claimed method corresponds to those named at page 10, lines 20-21; specifying observation of at least three of these locations is consistent with the simultaneous observation of the intact cell using the methods disclosed. These amendments were made to incorporate the language of allowed claims in the related application 10/713,234.

Claim 8 was amended for clarity, inserting the phrase 'at least one of' to ensure consistency with claim 7, which can include more than one signal transduction protein.

Claims 9 and 10 were amended only to provide consistent usage of the term 'localization patterns' and to make the verb plural, so it is consistent with the reference to two or more patterns.

Claims 11 and 12 were amended to be consistent with amended claim 7, in reciting 'intracellular localization patterns' rather than 'translocation'. This is believed to clarify the description and is supported by the specification which refers to intracellular localization patterns repeatedly, for example on page 5, lines 20-21.

Claim 20 was rephrased to address certain issues raised by the Office; nevertheless, it is intended to describe substantially the same process as original claim 20 but expressly using *toxic* compounds, and optimizing only the set of proteins used. The use of toxic compounds to provide a baseline or standard of comparison is described on pg. 10, line 22 to pg. 11, line 7. The terminology used in claim 20 was made consistent with that used in the other claims, and inherent steps such as contacting cells with toxic compounds were made explicit. The amended version omits the steps of refining the set of compounds: it only optimizes the set of proteins being used, which is consistent with the preamble of the claim and is supported by the specification at page 6, which describes "expanding the protein panel under inspection ..." without reference to expanding the set of toxic compounds in use; this it adds no new matter.

New dependent claims 21 and 22 claim subject matter that has been removed from claim 7 by the current amendment. Therefore, they also add no new matter.

The current amendments add no new matter, and address the Examiner's objections to various allegedly indefinite language by more precisely describing the claimed subject matter. Entry of the above amendments and reconsideration in light of these amendments and the following comments is thus respectfully requested.

Rejections based on 35 U.S.C. § 112, second paragraph.

1. The Office alleges that claim 7 (the rejection says claim 1, but it is clearly directed to claim 7, which is the first independent claim) is indefinite because the preamble, reciting a database does not correlate with the outcome, which describes a computer-readable collection of observations. The claim has been amended, but it retains the same basic structure and describes the same overall method, so the amendment alone may not remove the objections raised. The applicant therefore respectfully traverses the rejection.

The applicant uses the term 'database' in a sense that is consistent with general usage of the term. See, e.g., Webster's Collegiate Dictionary, 10th ed., which defines 'database' as "a usu. large collection of data organized esp. for rapid search and retrieval (as by a computer)." A collection of information is a database if it contains data that is organized to be retrievable, and implicitly the collected data must be united by some common function or purpose. Here, the collection of protein localization profiles has at least one purpose, it can be used for comparison to compounds having unknown toxicological properties, to enable one to predict what toxicity those compounds are likely to possess. Each profile in the collection contributes to that purpose by providing information about the protein localization pattern for one signal transduction protein in one type of cell in response to one particular toxic compound. The manner of accessing, querying or visualizing the profiles is not important in order to qualify as a database; only a uniting purpose for the collected data, sufficient organization so that it can be used for its intended purpose, and the ability to retrieve data.

A database is not a software package and need not have complex architecture, function or organization. It is an organized collection of data. One of ordinary skill would have no difficulty in creating a functional database populated by data produced by the method described in claim 7, perhaps with the use of a commercial database management software package. Thus the database mentioned in the preamble of claim 7 is consistent with the result achieved by the method of claim 7, with the understanding that one practicing the method of claim 7 would store the data produced by the method in some organized fashion. Practicing the claimed method produces intracellular

localization data for a multiplicity of signal transduction proteins. The accumulated data, if stored as described in computer-readable format, can constitute a database without any need for other "elements, objects or data structures" beyond those familiar to one of ordinary skill in the art. The applicant is not required to describe that which is well known in the art, which includes how a database software package can be used to store data in a database. The applicant thus asserts that the result of practicing the claimed method is production of data describing protein localization patterns, that one of ordinary skill would know how to record and format such data into a useable database, and that this indefiniteness rejection can properly be withdrawn.

Rejections based on 35 U.S.C. § 112, second paragraph.

2. The Office asserts that in claim 7, "observing" and "recording" require a continuity of action, which renders "optionally as a function of time" indefinite. The claim has been amended to remove the term "observing", but the term "recording" is still present, and the phrase "as a function of time" has merely been moved new to dependent claim 21. Thus the applicant traverses this rejection as it applies to those terms.

'Recording' (or indeed 'observing') can be either continuous or effectively instantaneous. The protein localizations of interest here, for example, could be recorded in a photographic image, as demonstrated by the Mochly-Rosen reference, which contains still images of localized proteins, or they could be captured in a video image, as discussed in the Gerdes & Kaether reference (both references were cited by the Examiner in this office action). A single 'snap shot' image would be consistent with the recitation of 'recording' the protein localizations at one time point; and recording as a function of time could, for example, be either recording a video image or recording a sequence of 'still shots'. Either way, the protein's intracellular localization would be captured as a function of time, and either way, the data for a compound known to be toxic could usefully be compared to similar data for a compound having unknown toxicity. Thus "recording" as a function of time is consistent with examples such as a video or a sequence of still images, and one of ordinary skill would understand how to practice the claimed method in either mode. Therefore, the applicant requests that this rejection be withdrawn.

Rejections based on 35 U.S.C. § 112, second paragraph.

3. The Office raised a number of objections and rejections based on the language of claim 20; that claim has now been amended. It is believed that each of the objections has been addressed by the amendment, and the phrases lacking antecedent basis have been amended to obviate the issue.

Most of the claim amendment involves rephrasing of the existing language in an attempt to more precisely explain and describe the subject matter, though the claim is now expressly drawn to a method using "toxic" compounds as previously stated. The term 'arbitrarily' has been retained, even though the Examiner alleges that it is indefinite. The applicant believes that it conveys to one of ordinary skill the fact that the selection process is not critical to the claimed invention and can be accomplished randomly or in any manner consistent with the interests or needs of the practitioner of the method. The claimed method can be successfully practiced regardless of the process used for selecting the initial set of proteins, because the iterative nature of the claimed process ensures that an appropriate set of signal transduction proteins will ultimately result.

The phrase "the range" is not amended, despite the Examiner's assertion that it is indefinite. That phrase is no more indefinite than the phrase "the group", which is commonly used to introduce a Markush group: in each case, the ensuing language completely describes what 'range' or 'group' is meant, and no antecedent basis is necessary to render it definite.

The Examiner also asserts that "marketed" is indefinite because "it is unclear how markets or marketing is incorporated into Applicant's invention." The applicant points out that 'the range of compounds marketed as small organic molecules" must be read as a whole and in context, and it expresses a concept that is clear in the context in which it is used. The phrase refers to a certain type of molecules, specifically those commonly referred to as 'small organic molecules' (or simply "small molecules"), which is a very familiar term in the chemical arts—especially in such fields as pharmaceuticals and toxicology. See, for example, the section entitled 'Small-molecule libraries' in "Combinatorial chemists focus on small molecules, molecular recognition, and

automation," Borman, Chem. Eng'g News, Feb. 12, 1996, available online at http://pubs.acs.org/hotartcl/cenear/960212/small.html **[attached as Exhibit A]**. It is well known in the chemical arts that 'small molecules' refers to a certain class of organic compounds, that those compounds are of special interest for certain purposes which include drug discovery, for example, and that those compounds are marketed for such purposes. The applicant thus believes that one of ordinary skill would understand the phrase "compounds marketed as small organic molecules."

The 'range' of such compounds, too, must be read in context: the claim refers to a set of proteins "which provides at least five principal components with respect to the range of compounds marketed as small organic molecules." The phrase "principal components" here would alert one of ordinary skill that a principal component analysis (PCA) is to be applied; "with respect to" indicates what kind of variables will be employed for this analysis. One of ordinary skill would understand from the claim language that 'the range of compounds' means that the variables to be used are those appropriate for the specified field (specifically, small organic molecules). Appropriate variables for describing small organic molecules, often referred to as 'molecular descriptors,' are also quite well known in the art. For an application of Principal Component Analysis (PCA) to a set of organic compounds, see "Predictive Carcinogenicity: A Model for Aromatic Compounds, with Nitrogen-Containing Substituents, Based on Molecular Descriptors Using an Artificial Neural Network", Gini, et al., J. Chem. Inf. Comput. Sci. 1999, Vol. 39, 1076-80 **[Exhibit B--abstract]**, applying PCA to the carcinogenicity of aromatic compounds having N-containing substituents, and using various types of molecular descriptors, including "electrostatic, topological, quantum-chemical, physicochemical, etc."

The Examiner also states that "principle [sic] components" is indefinite. However, the applicant notes that the claimed method is an optimization process, and one of ordinary skill would recognize the term "principal components" to indicate that principal component analysis (PCA), a routine statistical method, is to be applied. The term 'at least five principal components' then provides a statistical endpoint for the optimization process that would be understood by those of ordinary skill: the iterative process is complete when the statistical test is satisfied.

The applicant also realizes that the descriptive language required in a patent claim can be complex and nuanced, and that changes such as those made to claim 20 may introduce unanticipated issues. Thus the applicant would welcome any suggestions from the Office on ways to enhance the clarity of the claim language utilized.

Rejections based on 35 U.S.C. § 112, first paragraph.

Claims 7-13 were rejected as allegedly not enabled. Specifically, the Office asserts that one of ordinary skill could not practice the claimed invention to make and use a database of protein localization profiles, because, "no reference is given to any database design or functional characteristics." The applicant respectfully traverses this rejection.

Claim 7 has been amended to more clearly describe the process claimed, but it still claims a method to create a database of protein localization patterns. The Office says that since no details of the design and functional characteristics of a database are provided, one of ordinary skill could not make and use a database based on the specification. The Office then provides a reference which describes the many considerations to be taken into account in "developing a suitable database system." Among these are query capability, data characteristics, logic design, and physical database design.

Webster's Collegiate Dictionary, 10th ed., defines 'database' as "a usu. large collection of data organized esp. for rapid search and retrieval (as by a computer)." The applicant points out that the database described and claimed in the current application may not necessarily be "a suitable database *system*" as described in the reference, in the sense of being a highly specialized *system* with extraordinary flexibility. It is a database intended for a specific purpose, and need only provide those minimal features that are essential to its purpose. Nevertheless, the claimed method produces a collection of data, and one of ordinary skill would understand how to organize it and make it searchable, perhaps using a commercial database management software package. Thus the description enables creation of a 'database' as defined in Webster's Dictionary.

One of ordinary skill would be able to produce data using the method described, and to organize that data for retrieval according to cell type, toxin, or signal transduction protein. The data could be organized and stored using any of a number of commercial database management software packages, whose operation need not be explained since it is within the ordinary skill in the art and requires no inventive step. The result might not be as flexible as the state-of-the-art 3-D image storage *system* described in the reference cited by the Office; but it would be a functional database that could achieve its basic purpose. Furthermore, the claim uses the transition term 'comprises', so it admits of further steps such as creation of a more complex data storage and retrieval system. The applicant asserts that the method provided would enable one of ordinary skill to create a database as that term is commonly understood, which would contain protein localization profiles generated by the claimed method; therefore, this rejection may also be withdrawn.

Rejections based on 35 U.S.C. § 102.

The Office asserts that the claimed invention (specifically claims 7, 9-10, and 13) is anticipated by Gerdes & Kaether, 389 FEBS Letters 44 (1996). The reference allegedly provides a method to obtain a database of signal protein transduction profiles in response to toxic compounds. The applicant respectfully traverses this rejection.

To establish a rejection for anticipation, the Office must show that every element in the claim is present in a single prior art reference. Here, the Office alleges that Gerdes & Kaether provides a method to obtain a database. Yet the reference does not even allude to the desirability to make a database, let alone disclose a method to obtain one. The Office points to two web sites mentioned in the reference that supposedly provide video clips of protein movements (one of the sites was not operational when tested); yet isolated video clips not united by a common purpose or utility and in no way organized or connected do not a database make. At a minimum, a database must have some organizational characteristics; none are recited for these two web sites. The reference describes the web sites only as "[t]wo examples in which a 'green' movie can be accessed in a public domain folder..."]. They are not described as organized either individually or collectively into one or two 'databases'; they are not described as 'searchable'; furthermore, they

are not even part of the cited reference. Thus they cannot anticipate the database described in the claims.

The Office further asserts that the reference describes signal transduction protein localization profiles, because the abstract says, "GFP can be used to localize proteins, to follow their movement..." Respectfully, this does not disclose localization of *signal transduction* proteins, thus it cannot anticipate the claimed method. While the reference discusses a number of proteins that have been tagged and followed using GFP, most of them appear to have been used to monitor physical movement of parts of a cell (tubulin / cytoskeletal apparatus (pg. 45, col. 1 ll. 3-9, and col. 2, last line, cont'd on pg 46); migration and phagocytosis (pg. 45, col. 1, second paragraph); formation and movement of transport vesicles (pg. 46, section 3.2 to 3.5) ; GFP-myosin "in the tips of retracting pseudopods" (pg. 45, col. 2, ll.3-4), or to track the movement of proteins across membranes (pg. 46, sect. 3.4). The claimed invention, on the other hand, relates to localization patterns of intracellular signal transduction proteins and how those patterns change in response to toxic compounds. Examples of such signal transduction proteins include protein kinase C (PKC) and other kinases involved in signal propagation within a cell; it is not obvious that any of the disclosed examples would qualify as signal transduction proteins.

The Office also suggests that the protein visualization as described by Gerdes & Kaerther relates to a "response to toxic compounds" because the reference uses the phrases "cAMP-stimulated" and "insulin-stimulated" to describe the cells being studied. The applicant points out that cAMP and insulin are not generally considered 'toxic' compounds: they are physiologically essential materials. Their effect on the cells presumably reflects normal regulation of the behavior of a cell rather than the presumptively different effect of a toxic compound. Thus the reference cannot anticipate a method providing protein localization profiles that result from exposure of cells to toxic compounds.

The Gerdes & Kaether reference does not provide a method to obtain a database, nor does it expressly describe monitoring the locations of signal transduction proteins, nor does it disclose the exposure of cells to toxic compounds, or observing the intracellular movement of

proteins in response to toxic compounds. Thus it does not anticipate the claimed invention, and this rejection may be withdrawn.

The rejections of the dependent claims must fall with the withdrawal of the rejection of the independent claim 7, since the dependent claims incorporate all of the limitations present in the independent claim they depend from. Nevertheless, the applicant points out that the rejection of claim 9 over the data in Table 1 of the reference overlooks the fact that in the claimed method, each of the 'at least two signal transduction proteins' being monitored would be compared in the presence of a common toxic compound; the reference does not disclose using the same compound on different cell types to compare their protein localization responses, let alone creating a profile of the changes in protein localization induced by such compounds, or the effect where such compounds are toxic. Likewise, the rejection of claim 10 overlooks the same fact: the multiplicity of proteins in the claimed method would be observed in the presence of each of the toxic compounds employed. In the reference, no common perturbation that would elicit a relevant response diagnostic of toxicity is applied to the various proteins mentioned.

Finally, as argued above, the mere reference to two computer-readable video clips somewhere on the internet does not comprise a 'database' within any ordinary meaning of that term. Thus the rejections for anticipation by the Gerdes and Kaether reference may be withdrawn.

Rejections based on 35 U.S.C. § 102.

The Office next alleges that Sawin & Nurse anticipates the invention of claim 20, because it allegedly describes identifying a set of signal transduction proteins, determining how their localization changes under the influence of a set of compounds, and refining the set of signal transduction proteins in response to the screening process to prepare a second set of such proteins and compounds; then iterating this process until a statistical endpoint is reached.

Claim 20 has been amended and the compounds are now described as toxic compounds. Furthermore, the steps relating to refining the set of compounds have been removed: the process

now refines only the set of proteins, not the set of compounds. Nevertheless, the applicant traverses the characterization of Sawin and Nurse as potentially relevant to the amended claim 20.

The Sawin and Nurse reference is entitled, "Identification of fission yeast nuclear markers using random polypeptide fusions with green fluorescent protein." As the title suggests, the reference involved *random* fusions of GFP to proteins: the actual proteins expressing or carrying the GFP were not even characterized, though partial DNA sequences for some of the mutants produced were obtained in an attempt to understand which proteins they represented. Thus the reference does not relate to or disclose any "set of signal transduction proteins", which is an element of the claim.

The Office suggests that Figure 2 shows changes in intracellular localization patterns "in response to an initial set of compounds." That "set of compounds" is equated to the "pSGA genomic library" of the reference. According to the reference, though, the localizations depicted in Figure 2 are determined by where the GFP DNA was randomly inserted into the pSGA genomic library, i.e., which DNA sequence the GFP sequence happened to join. The localization is determined not by the effect of an external compound on the cell, but by the random process of gene insertion. Thus the localization observed is apparently that which is natural for the expressed protein carrying the GFP; it has nothing to do with the effect of an added compound and there is nothing to even suggest that it is affected by a 'toxic compound.'

Also, the pSGA genomic library indeed is a set of compounds, but it is not used to affect the localization of proteins in cells. Rather, it is used to introduce GFP labels into the cells that are randomly associated with various DNA sequences and thus are expressed as randomly created fusion proteins. The pSGA library is therefore not analogous to the compounds described in original claim 20 or to the toxic compounds described in amended claim 20. It is a collection of DNA that is used to randomly generate new cell types that contain one or more GFP-labeled proteins, and Figure 2 shows where those randomly selected proteins naturally reside rather than providing any information about how an added 'toxic compound' affects protein localizations.

Similarly, the Office recites the screening of colonies of cloned cells as analogous to the screening steps of the claimed method. Yet the screening in Sawin and Nurse is to eliminate those colonies that failed to express a fluorescent GFP-containing protein. The screening steps in the claim are to eliminate from further consideration those particular signal transduction proteins that are not affected by added compounds in a way that provides useful information about how the added compounds affect the cell. Also, the "homologs" that the Office suggests amount to a second set of proteins are merely the DNA sequences from other organisms that most nearly match a partial sequence of the plasmid that produced one particular clone; *they are not necessarily even expressed proteins*, only DNA sequences. See Sawin, pg. 15147, col. 2: "These two genes have been isolated in a wide variety of genetic screens..." Finally, the "identified markers" which the Office equates to the 'principal components' of the original claim 20 are also inapt. The markers represent expressed proteins in clones that are of interest to the researchers because those clones allow the researchers to see particular subcellular structures. See, e.g., the title: Identification of fission yeast nuclear markers using random polypeptide fusions with green fluorescent proteins." The 'principal components' of original claim 20 are statistical correlations, not physical or chemical entities.

Most of the allegedly anticipatory elements of this reference are not even analogous to the claim element to which they were compared by the Office. The applicant thus asserts that this reference is irrelevant to patentability of any claim in this application. Specifically, it fails to disclose any set of signal transduction proteins, any set of toxic compounds, or any step where protein localization patterns in a cell are affected by toxic compounds. It thus cannot anticipate claim 20, and withdrawal of this rejection is requested.

Rejections based on 35 U.S.C. § 103.

The Office rejects claims 1 (again, this is presumed to mean claim 7), 8 and 10 over Gerdes & Kaether in view of Mochly-Rosen, 268 Science 247 (1995).

The applicant first points out that the references are arguably non-analogous art: the claimed invention relates to a way to predict toxicity of unknown compounds by determining how

various toxic compounds affect the localization of signal transduction proteins within a cell. See, e.g., Specification, pg. 5, ll.6-10. The references relate to "Green fluorescent protein: applications in cell biology" (Gerdes & Kaether) and "Localization of Protein Kinases by Anchoring Proteins: A Theme in Signal Transduction" (Mochly-Rosen). The references thus relate to the functioning of a cell under ordinary conditions. Neither reference relates to or suggests that the localization of signal transduction proteins can be used to detect, characterize, or predict the effect of potentially toxic compounds on cells. Nothing in the references suggests using the teachings of either reference to characterize the effects of toxic compounds on cells or on protein localization.

Furthermore, the references cannot support an obviousness rejection, because they do not disclose all of the elements of the claimed invention. As discussed above, Gerdes & Kaether does not describe a method to obtain a database at all, let alone one based on the effects of toxic compounds. The reference merely points to two web sites supposedly having videos of cells where protein locations are visualized using green fluorescent protein (GFP). Indeed, it does not suggest using protein localization data to understand the effects of exogenous compounds generally, let alone the effect of toxic compounds in particular. Its conclusion predicts two areas for increased application of GFPs: genetic engineering to produce new GFP analogs, and studying protein-protein interactions using doubly-mutated cells. Gerdes & Kaether, last paragraph. Neither of these is relevant to the claimed method.

Mochly-Rosen is no more on point: it describes research on anchoring proteins, which tend to hold signal transduction proteins in place, and suggests that one way to disrupt signaling would be to inhibit the binding of a signal transduction protein to its anchoring protein. See Mochly-Rosen, abstract. It does not relate to detection or characterization of toxic compounds by analyzing the translocation of proteins inside cells exposed to such toxins. The Office says that Mochly-Rosen shows that "serine-threonine kinases may be useful in the development of therapeutic agents." But it is unclear why that is relevant: the claimed invention does not relate to therapeutic agents, the claims are drawn to effects of toxic compounds on cells and to a database of information on such effects. Furthermore, the reference to development of therapeutics in Mochly-Rosen is to "inhibitors of the interaction between the kinases and their anchoring proteins." Id. The

reference is not relevant to observing or predicting the effects of toxic compounds on intracellular protein localizations, since the inhibitors of the reference are expressly therapeutic agents rather than toxins.

Furthermore, in addition to establishing that the cited references disclose all of the elements of the claimed invention, to establish a *prima facie* case for an obviousness rejection the Office must also show that one of ordinary skill would be motivated to combine the cited references and would have a reasonable expectation of success in doing so. Here, the Office has not met that burden. The Office points to no reason one would combine the teachings of Gerdes & Kaether with those of Mochly-Rosen to arrive at the claimed invention; only that one would be motivated to combine their teachings because both references "teach the use of GFP as a powerful tool for uncovering dynamic cellular events." Their only commonality seems to be the use of GFP as a tool to monitor locations of proteins in cells. That technique is one that *may* be used in the invention, but it is not essential to the broad scope of the invention as claimed in claim 7. Neither reference teaches or suggests that the localization of proteins is even related to the toxic effects of added compounds. Neither reference relates to or suggests the study of toxic compounds, nor does either relate to or suggest the use of protein localization as a predictive tool for responses to unknown compounds. The Office's reference to "uncovering dynamic cellular events" is far too vague to suggest the claimed invention. Thus neither reference provides motivation to practice the methods of the invention, and neither reference suggests a utility for a database produced by the claimed method. Consequently the combination of references cannot render the invention obvious, and this rejection should be withdrawn.

Rejections based on 35 U.S.C. § 103.

Claims 7 (again, the Office writes 'claim 1' but is presumably referring to claim 7) and 11 are rejected over Gerdes & Kaether in view of Gerhard (US Patent No. 5,684,628). The applicant notes that Gerhard is only potentially relevant to claim 11; claim 7 does not require or describe any use of a wide-field microscope that would render Gerhard relevant for an obviousness rejection. Absent the requirement to use a microscope, one would not be motivated to combine

Gerdes & Kaether with the teachings of Gerhard, so Gerhard is not relevant to patentability of claim 7. And Gerhard does not add anything substantive to Gerdes & Kaether other than the use of the wide-field microscope. As argued above, Gerdes & Kaether fails to teach or even suggest elements of the claimed invention, including the use of toxic compounds, the use of signal transduction protein localization data, and the creation of a database of protein localization profiles. Gerhard is a patent covering a microscope stage; while useful for viewing of individual cells, it does not provide or suggest those elements of claim 7 that Gerdes & Kaether fail to disclose. Thus this combination of references does not render the claimed invention obvious, and this objection can be withdrawn.

Nevertheless, to avoid any confusion, the applicant points out that the phrase, "integrate that information over time" from Mochly-Rosen, which the Office alluded to, should not be confused with the phrase in the claims, "as a function of time". Integration over time is a means of collecting data on a single static image over time so that weak signals can be accumulated over time without increasing the potentially-destructive illumination; the result is a single image of a stable system. 'As a function of time' in the claims refers to discrete observations or records of a dynamic scenario made at intervals to observe the changes occurring therein.

Since neither reference suggests or relates to the claimed invention, "a method to obtain a database of signal transduction protein localization profiles in response to toxic compounds", the Office has not met its burden of establishing a *prima facie* case of obviousness. One attempting to solve the present problem, predicting the toxicity of unknown compounds, would not have been motivated to combine the teachings of the cited references. Nor would one have had a reasonable expectation of arriving at the claimed invention by doing so, since nothing in the references teaches or suggests such a use for GFP or for signal transduction proteins. The combination of references does not teach or suggest all elements of the invention, nor does either directly relate to or suggest the claimed invention. Thus this rejection may also be withdrawn.

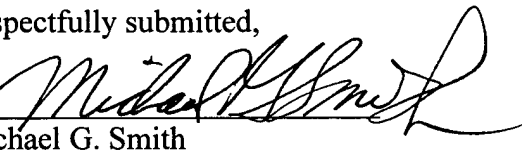
CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no.*. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: December 16, 2004

Respectfully submitted,

By 

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SPECIAL REPORT

Combinatorial chemists focus on small molecules, molecular recognition, and automation

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Drug candidates traditionally have been synthesized one at a time, a time-consuming and labor-intensive process. But many researchers in academia, government, biotechnology firms, and drug companies increasingly are turning to combinatorial chemistry - a strategy for creating new drugs that, it is hoped, will speed the drug discovery process significantly.

The idea of combinatorial chemistry is to make a large number of chemical variants all at one time; to test them for bioactivity, binding with a target, or other desired properties; and then to isolate and identify the most promising compounds for further development.

The success of combinatorial chemistry is still uncertain. No drugs discovered combinatorially have been approved for marketing, although several are currently in development. But many researchers believe the technique will prove to be an efficient and cost-effective tool for identifying new medicines.

In combinatorial chemistry experiments, chemical libraries (large collections of compounds of varied structure) are produced by sequentially linking different molecular building blocks, or by adding substituent "decorations" to a core structure such as a polycyclic compound. Libraries may consist of molecules free in solution, linked to solid particles or beads, or even arrayed on surfaces of modified microorganisms.

Combinatorial chemistry initially focused on the synthesis of very large libraries of biological oligomers such as peptides and oligonucleotides. But drug developers generally prefer to focus on small organic molecules with molecular weights of about 500 daltons or less - the class of compounds from which most successful drugs have traditionally emerged. So combinatorial chemistry researchers are concentrating on small organic compounds as well.

Drug discovery is the primary goal of most combinatorial chemistry research, but combinatorial methods also have potential applications for development of advanced materials and catalysts.

One of the challenges of combinatorial chemistry is the difficulty of identifying "hits" (active compounds) present at vanishingly low concentrations in complex combinatorial libraries. To address this problem, ingenious encoding schemes have been developed. Two groups have independently

developed the latest concept in this field - radiofrequency encoding, in which information about library compounds is stored on microchips.

Instrumentation systems to help speed combinatorial chemistry experiments have been developed in-house at a number of biotechnology and pharmaceutical companies. And several combinatorial automation systems are available commercially or undergoing intensive development.

Combinatorial chemistry has come a long way in just a few years, but further advances are needed and new applications are anticipated. Directions in which the field is headed range from combining combinatorial chemistry with computational drug-design strategies to the use of combinatorial molecular recognition for studies of protein function.

Creating libraries

Combinatorial libraries are created in the laboratory by one of two methods - split synthesis or parallel synthesis. In split synthesis, compounds are assembled on the surfaces of microparticles or beads.In each step, beads from previous steps are partitioned into several groups and a new building block is added. The different groups of beads are then recombined and separated once again to form new groups. The next building block is added, and the process continues until the desired combinatorial library has been assembled.

Before split synthesis was developed, explains chemistry professor Kim D. Janda of Scripps Research Institute, La Jolla, Calif., "people created diversity using mixtures of compounds. In a coupling step, you would add, let's say, reagents A, B, and C in one pot, and A, B, and C would all compete to become integrated at the same site. But in doing that you can have problems with kinetics. One reaction may be faster than another and you may not get equal distribution of the three components."

Split synthesis "got away from all that," says Janda. "You could create diversity using separate reactions, so the components would have an equal chance to add in to a site, and then by mixing compounds together again you got the diversity you needed."

Libraries resulting from split synthesis are characterized by the phrase "one bead, one compound." Each bead in the library holds multiple copies of a single library member. Split synthesis greatly simplifies the isolation and identification of active agents because beads (and implicitly individual library members) are large enough to be observed visually and separated mechanically.

Combinatorial libraries can also be made by parallel synthesis, in which different compounds are synthesized in separate vessels (without remixing), often in an automated fashion. Unlike split synthesis, which requires a solid support, parallel synthesis can be done either on a solid support or in solution.

A commonly used format for parallel synthesis is the 96-well microtiter plate. Robotics instrumentation can be used to add different reagents to separate wells of a microtiter plate in a predefined manner to produce combinatorial libraries. Hits from the library can then be identified by well location.

Split synthesis is used to produce small quantities of a relatively large number of compounds, whereas parallel synthesis yields larger quantities of a relatively small number of compounds. And split synthesis requires that assays be performed on pools of compounds, whereas assays on individual compounds can be run on libraries created by parallel synthesis. While slower, testing individual compounds is sometimes advantageous because serious interferences and complications can arise when multiple compounds are tested simultaneously.

A special case of parallel synthesis is spatially addressable synthesis, pioneered by researchers at Affymax Research Institute, Palo Alto, Calif. In this technique, libraries are synthesized in arrays on microchips, and all the compounds on a chip are assayed simultaneously for binding or activity. Hits can then be identified by the piece of real estate they occupy on the chip. Using a chip-making technique called photolithography, Affymax researchers have generated arrays of more than 65,000 compounds on chips about 1 sq cm in area.

Bioactive combinatorial compounds synthesized by split synthesis can also be identified by deconvolution, a technique in which each variable position in a compound library is tested to find the building block that makes the strongest contribution to activity at that site.

Solid-phase and solution-phase combinatorial synthesis each have their advantages and disadvantages. Solid-phase synthesis permits use of excesses of reagents to drive reactions to completion, since excess reagents can be washed away from beads very easily afterward. However, solution-phase synthesis is more versatile because many organic solution-based reactions have not been adapted for solid-phase work.

Janda and coworkers at Scripps recently developed a liquid-phase synthesis procedure that combines some of the advantages of solution-phase and solid-phase synthesis [*Proc. Natl. Acad. Sci. USA,* **92,** 6419 (1995)]. The procedure involves use of polyethylene glycol monomethyl ether in place of solid-phase beads as a foundation for combinatorial assembly. The polymer is soluble in a variety of aqueous and organic solvents, making it possible to use solution-phase combinatorial synthesis. But the polymer can be precipitated out of solution by crystallization at each stage of the combinatorial process to facilitate purifications.

Small-molecule libraries

Combinatorial chemistry began with the synthesis of large libraries of biopolymers such as peptides and oligonucleotides. In some cases, these were created on surfaces of genetically modified microorganisms, such as bacteriophage particles, by inserting combinatorial DNA oligomers into genes that encode cell-surface proteins.

However, peptides and oligonucleotides are problematic for drug development because their oral bioavailability is poor and they are degraded rapidly by enzymes. Hence, the focus of combinatorial research has shifted in recent years to libraries of nonpolymeric small molecules having molecular weights of about 500 daltons or less.

In a pioneering study, chemistry professor Jonathan A. Ellman and coworkers at the University of California, Berkeley, synthesized the first such library by creating variants of benzodiazepines, a class of compounds that has been a fertile source of successful drugs [*J. Am. Chem. Soc.,* **114,** 10997 (1992)]. Since then, researchers have found ways to synthesize combinatorial libraries based on many other classes of small organic compounds.

A recent example is work by Mark A. Gallop, director of combinatorial chemistry, and coworkers at Affymax. They used a cycloaddition reaction to prepare a small-molecule combinatorial library of about 500 mercaptoacyl prolines [*J. Am. Chem. Soc.,* **117,** 7029 (1995)]. By screening this library, they identified an unusually potent inhibitor of angiotensin-converting enzyme (ACE). ACE inhibitors are used as treatments for hypertension and heart disease.

And the group of Stephen W. Kaldor, head of combinatorial chemistry research at Eli Lilly & Co.,

Indianapolis, in collaboration with scientists in Lilly's central nervous system (CNS) group, has used combinatorial chemistry to identify an orally active CNS agent by combinatorial optimization of an existing lead. The low molecular weight nonoligomeric drug candidate entered clinical trials in November. "This is one of the first small-molecule combinatorial compounds to go into humans," says Kaldor.

A major challenge of small-molecule combinatorial chemistry has been to adapt conventional solution-phase organic reactions to reactions on solid-phase particles. Ellman says one of his group's efforts "has been to expand the kind of chemistry that can be performed on solid supports in a simultaneous synthesis format - in particular, carrying out different types of carbon-carbon bond-forming reactions. For example, we've developed general enolate alkylation conditions where side reactions that can be a problem in solution don't occur."

Paralleling the increasing use of small-molecule libraries is a trend toward assaying libraries having smaller numbers of components. "People seem to be much more comfortable working with smaller mixtures - probably a hundred components or less in a mixture, rather than the mixtures of 10^5 and 10^6 compounds per pool that we saw in the early experiments," says Ronald N. Zuckermann, associate director of bioorganic chemistry at Chiron Corp., Emeryville, Calif. "The lower the number of compounds, the more confidence you can have in the biological data" because artifacts arise more readily in the screening of large pools of compounds.

Ellman agrees that "people have gotten away from screening really large mixtures of compounds. They either want to screen them individually or in smaller pools of under 100 compounds. It's easier to extract out binding data in that format."

Oligomers and materials

Carbohydrates have lagged behind other types of compounds in combinatorial library development because of the complexity of oligosaccharide chemistry, but carbohydrate libraries are now beginning to appear. For example, Ole Hindsgaul and coworkers at the department of chemistry of the University of Alberta, Edmonton, in collaboration with researchers at the University of Georgia, Athens, and Ciba Central Research Laboratories, Basel, Switzerland, have developed a "random glycosylation" strategy for making oligosaccharide libraries in solution [*Angew. Chem. Int. Ed. Engl.*, **34,** 2720 (1995)]. They produced a library of all 18 possible fucosylated trisaccharides from disaccharide precursors.

And at a recent meeting, chemistry professor Daniel E. Kahne of Princeton University reported construction of the first solid-phase carbohydrate library, using chemistry for solid-phase synthesis of oligosaccharides developed earlier by his group. This technique has been licensed to Transcell Technologies, Monmouth Junction, N.J. In preliminary work, compounds isolated from one carbohydrate library have been shown to bind a carbohydrate-binding protein with greater affinity than the protein's natural ligand. "Carbohydrates play a central role in some very important biological processes, so having access to libraries of these compounds is critical," says Kahne.

Another type of oligomer being pursued combinatorially is peptoids, peptide analogs that are not recognized by peptide-cleaving enzymes. Chiron researchers recently discovered a candidate urokinase receptor antagonist from a peptoid library, and the compound is currently in preclinical studies as a potential anticancer agent.

"One of the primary advantages of peptoids is their synthetic accessibility," says Zuckermann. "They are efficiently synthesized by the submonomer method, which uses primary amines and bromoacetic acid as

starting materials - both very cheap, and there are literally thousands of amines readily available. The combination of this chemistry with robotic synthesis has led to a truly high throughput synthesis facility."

Chiron's identification of nanomolar peptoids that bind to transmembrane receptors [*J. Med. Chem.*, **37,** 2678 (1994)] "was the first example of the discovery of potent ligands to pharmaceutically relevant receptors from a combinatorial library of nonpeptides or nonnucleic acids - that is, synthetic compounds," Zuckermann adds. "I believe that this work helped inspire others to continue to move away from peptides and further toward small molecules."

Combinatorial chemistry can also be extended entirely beyond the realm of organic chemistry. For example, physicist Xiao-Dong Xiang of Lawrence Berkeley National Laboratory, chemistry professor Peter G. Schultz of UC Berkeley, and coworkers recently devised a combinatorial strategy for finding advanced materials with novel chemical or physical properties - extending "the combinatorial approach from biological and organic molecules to the remainder of the periodic table," as they put it [*Science,* **268,** 1738 (1995)].

Xiang, Schultz, and coworkers used thin-film deposition and physical masking techniques to synthesize libraries of solid-state materials. The properties of the resulting materials were then evaluated to identify promising candidates for further development.

Encoding

In spatially addressable combinatorial synthesis, active compounds can be identified by location. But in other forms of combinatorial chemistry, identifying hits is not so easy because there's often too little of each compound present for characterization with traditional analytical chemistry techniques.

Hence, many researchers now use some form of tagging or encoding to label compounds in large combinatorial libraries. The first such encoding scheme was proposed in 1992 by Scripps President Richard A. Lerner and molecular biologist Sydney Brenner at the institute. They suggested that a combinatorial library could be encoded with oligonucleotides synthesized in parallel with library compounds and linked to each one. Amplification or decoding of the attached oligonucleotide would serve to identify the small molecule bound to each bead.

This idea was independently arrived at and reduced to practice by scientists at Affymax. Later on, researchers at Chiron and at Selectide, Tucson, Ariz., developed similar techniques in which peptides instead of oligonucleotides were used as the sequenceable encoding oligomers.

In 1993, chemistry professor W. Clark Still and coworkers at Columbia University developed a second major type of encoding scheme, in which chromatographically resolvable organic tags were used as encoding elements for bead-based combinatorial libraries. Still devised the technique in response to concerns about the tendency of DNA and peptide tags to break down under the often very rough conditions of organic synthesis.

In Still's technique, inert halogenated aromatic compounds are used to encode the chemical reaction history experienced by each bead. These tags are identified by capillary gas chromatography to reveal the identity of active compounds in the library. Kahne, who used this type of encoding to construct his combinatorial carbohydrate library, says the method "is as good as it gets for identifying hits - a very simple solution to a very important problem."

The most recent development in encoding technology involves the use of radiofrequency tags. Chemistry professor K. C. Nicolaou at Scripps and the University of California, San Diego, together with senior chemist Xiao-Yi Xiao, President and Chief Executive Officer Michael P. Nova, and their coworkers at IRORI Quantum Microchemistry, La Jolla, Calif., developed a technique in which memory devices are associated or coated directly with derivatized polymer during combinatorial synthesis [*Angew. Chem. Int. Ed. Engl.*, **34,** 2289 (1995)]. The chips encode relevant information about the synthetic pathway - including not only reagents used, but also reaction conditions such as temperature and pH. The device can then "report" this information to a receiver via radiofrequency transmission.

"We're putting a manual system to do this type of radiofrequency combinatorial chemistry out on the market in March," says Nova. The system will include radiofrequency memory devices in MicroKans, tiny spherical capsules with porous walls that also enclose polymer beads for combinatorial synthesis.

A related technique was developed independently by synthetic chemist Edmund J. Moran and coworkers at Ontogen Corp., Carlsbad, Calif., and the University of California, Los Angeles [*J. Am. Chem. Soc.*, **117,** 10787 (1995)]. This approach differs from the Scripps technique in that reaction data from each stage of combinatorial synthesis are stored in a computer database, rather than being retained in the chip itself. An identification number stored in the memory of each chip is a pointer to reaction information in the database. Moran and coworkers have applied the strategy successfully to the discovery of novel inhibitors of a protein tyrosine phosphatase.

Molecular recognition

A combinatorial chemistry application that has become increasingly active in the past year or so, and that promises to grow even more rapidly in the future, is combinatorial molecular recognition - the use of combinatorial techniques to study binding between biological or synthetic receptors and their ligands. Researchers in the combinatorial molecular recognition community "want to be able to make small molecules that do the kinds of things that antibodies do - tightly and selectively bind important molecules or transition states," explains Columbia's Still.

"We made libraries of substrates just to measure the binding properties of compounds synthesized as enantioselective receptors," says Still, "and the receptors did indeed have significant sequence-selective binding properties that had never been observed before." The results of such experiments suggest, says Still, "that virtually anything people can do with antibodies ought to be doable with small molecules, and that it may not be that hard to identify small molecules that are as selective as antibodies for binding substrates."

Chemistry professor Stuart L. Schreiber and coworkers at Harvard University are also using combinatorial molecular recognition - in this case in conjunction with nuclear magnetic resonance spectroscopy (NMR) - to study protein receptors. They have focused initially on the SH3 domain, a frequently occurring structural feature in proteins (such as tyrosine kinases) involved in signal transduction.

The researchers identified peptide ligands that bound SH3 in two binding pockets that make up part of the SH3 binding site. The SH3 binding site also includes a third binding pocket that is highly variable in structure and is therefore referred to as a "specificity pocket." A combinatorial strategy led to the discovery of two classes of peptide ligands that bind to the three pockets in opposite orientations, as determined by NMR analysis of the SH3-ligand complexes. Last month, Schreiber and coworkers reported also having identified nonpeptide elements that bind to the specificity pocket [*J. Am. Chem. Soc.*, **118,** 287 (1996)].

Chemistry professor Fredric M. Menger and coworkers at Emory University, Atlanta, are also using a form of combinatorial molecular recognition - in this case to identify industrial catalysts [*J. Org. Chem.*, **60,** 6666 (1995)].

"Libraries have in the past been screened for noncovalent binding," says Menger. "But this is the first, or one of the first, cases where purely organic libraries have been investigated for catalytic activity. We make hundreds or thousands of compounds very quickly and then test their catalytic power. The potential catalysts are polymers that have multiple functional groups in different proportions and different sequences, plus a metal ion."

In screening for catalytic activity, "we selected the hydrolysis of a phosphate ester," says Menger, "but one could choose any reaction of interest. Once a polymer with activity is found, we begin tinkering with the proportions to fine-tune it until it gets faster and faster."

Using this approach, Menger and coworkers have identified polymers that accelerate phosphate hydrolysis by a factor of 10^4 or more. According to Menger, "The potential exists for even greater acceleration ... since only a small portion of the vast number of possible combinations has as yet been tested."

In future work, the researchers hope to make chiral polymers that can reduce functional groups enantioselectively. "I would not be surprised if in 10 years most new catalysts are developed combinatorially," says Menger. "Industry is moving more and more toward aqueous systems to avoid organic solvents. If one could devise catalysts for organic reactions in water, that would be a useful practical development."

Automation

Planning and performing combinatorial experiments in the laboratory is a complex and potentially tedious process. Hence, "A future trend is going to be greater availability of automation devices," says Chiron's Zuckermann. "A lot of solutions are being developed for automating combinatorial split synthesis or multiple parallel synthesis."

For example, Chiron has developed proprietary robotic combinatorial synthesizers. "We now have third-generation units working in our labs that feature all-glass reaction vessels, heating to 120°C, and flexible software, [allowing] automation of most organic reactions," says Zuckermann.

Ontogen has developed OntoBLOCK, an in-house combinatorial chemistry automation system that can produce 1,000 to 2,000 small organic molecules per day by parallel array synthesis. The system includes reaction blocks containing 96 reaction vessels, from which compounds can be transferred directly to standard 96-well microtiter plates for high-throughput screening.

Bohdan Automation Inc., Mundelein, Ill., markets a combinatorial chemistry reaction block that accommodates a wide variety of organic solvents and handles both solid-phase and solution-phase chemistry. Advanced ChemTech, Louisville, markets instrumentation for combinatorial peptide and organic synthesis. And Tecan U.S. Inc., Research Triangle Park, N.C., offers an organic chemical synthesizer called CombiTec that includes a robotic sample processor and reaction blocks of eight to 56 chambers.

Robotics maker Zymark Corp., Hopkinton, Mass., has put together several different automation systems that enable their clients to do solution-phase combinatorial synthesis and solid-phase peptide and

peptoid synthesis. The reactions can generally be performed under inert gas at a variety of temperatures.

According to Brian Lightbody, general manager of drug discovery business development at Zymark: "The process of generating combinatorial compounds involves several steps in addition to the actual reaction - [including] initial formulation of the reactants, labeling, pooling and splitting, cleavage, liquid-liquid extraction, solid-phase extraction, and evaporation. These steps require extensive manual labor. ... An automated robotic approach can often be implemented to fulfill these requirements, dramatically reducing the manual labor and eliminating the sources of human error."

A combinatorial chemistry system still in the prototype stage is the Nautilus, a synthetic chemistry workstation being developed by Argonaut Technologies Inc., San Carlos, Calif. The instrument handles a wide range of reagents, with capabilities for temperature control and use of inert atmospheres.

"The Nautilus allows you to do pretty much what you're able to do on the bench except in an automated fashion," says Argonaut President and CEO Joel F. Martin. "The system is completely enclosed and encapsulated, with a pressurized fluid delivery system and no exposure to the atmosphere whatsoever. It's a closed system, and all wetted surfaces within the instrument are glass or [polytetrafluoroethylene, such as DuPont's] Teflon."

Procedures that have been demonstrated on the Nautilus include a Suzuki coupling (a carbon-carbon bond-forming reaction at elevated temperature using an air-sensitive palladium catalyst), a butyllithium reaction, enolate reactions of the type developed by Ellman and coworkers, and synthesis of a solid-phase druglike molecule. "We chose tough organic reactions that no one would ever have conceived of doing in an automated synthesizer in the past," says Martin. The Nautilus is scheduled to be released commercially in August.

CombiChem Inc., San Diego, is developing commercial instrumentation for combinatorial chemistry that is likely to be competitive with the Nautilus. "Every company now is looking at ways of automating synthesis, purification, and analysis," says CombiChem Chief Operating Officer Peter L. Myers. "There's a major revolution going on. It's probably not obvious to a lot of people, and the academics may think we're overemphasizing it. But I know for a fact that every company now is looking to automate combinatorial chemistry because chemistry's become the rate-determining step."

As to whether conventional robotics instrumentation can be used effectively for combinatorial chemistry synthesis, "This immediately gets one into a debate," replies Myers. "When you're doing chemical reactions to make small molecules, many of the reactions are sensitive to conditions such as the presence of water vapor or oxygen, so inert atmospheres such as argon and nitrogen are often needed. The only successful way of blanketing a reaction is to have a closed system - one that is sealed. And if you seal it, then of course you can't use a robot very easily."

Myers adds, "This is why we, and also Argonaut, have gone to nonrobot systems - closed systems that work on valves and plumbing. ... That essentially means individual reaction vessels presealed with a solid support or chemicals inside, delivery by valving, and some way to agitate or stir the contents. Then you let the reaction proceed and wash the resin at the end, if it's a solid-phase reaction." However, he concedes that many researchers are currently using robotic systems instead of closed systems for combinatorial synthesis, "so the jury is out on which is the most acceptable."

CombiChem's instrument will be capable of automating both solid-phase and solution chemistry. "If you really want to exploit as much diversity as you can ... you have to be able to do something in addition to just solid-phase chemistry," says Myers. "The reason is pretty obvious. There are about 150 reactions

now that work on solid phase. Some of those reactions work extremely well, some are still very poor yielding. But the organic chemists have an armory of thousands of chemical reactions that have been developed over the years, and of course primarily most of those are done in solution."

3-Dimensional Pharmaceuticals Inc., Exton, Pa., is also developing combinatorial chemistry instrumentation. The system is based on a technique called DirectedDiversity, an iterative optimization process that explores combinatorial space through successive rounds of selection, combinatorial synthesis, and testing. In each step, a chemical library is generated by robotic instruments, structure-activity information is obtained on library members, and data are analyzed to determine how closely the synthesized compounds match a set of desired properties. In each succeeding iteration, the structure-activity models are refined and new compounds are created until desired drug leads have been identified.

Future needs and prospects

Combinatorial chemistry has come a long way in the past few years, but many challenges still lie ahead. For example, Ellman foresees further development of solid-support chemistry, including new linkage strategies and novel methods for synthesizing support-bound libraries and cleaving compounds from supports. "And people will continue to focus on different types of templates - novel templates for the versatile display of functionality," he says.

Ellman also believes "there are some interesting opportunities in the area of combining combinatorial strategies with computational strategies and structure-based design. The idea is to use information about three-dimensional structures of receptors and enzymes in combination with libraries to rapidly identify high-affinity ligands. It is going to be interesting to see how best to combine these two approaches." The recent SH3 study by Schreiber's group exemplifies this strategy of using a knowledge of protein and protein-ligand structure to help design optimal libraries.

Eric M. Gordon, vice president of research and director of chemistry at Affymax, points out that "some people enter into the molecular diversity sphere with the idea that it's a random process and that what you want to do is make as many molecules as you can that are as different from each other as they can be, but that no particular thought has to go into the design of these libraries. That's an extreme position. I believe that combinatorial chemistry doesn't stand alone. It should be integrated in with the arsenal of tools used for drug discovery, as opposed to being viewed as a competitive technology, say with structure-based design. I think the fate of it and the greatest power of it is going to be when it's used in concert with structure-based approaches and computational approaches."

Still believes that future prospects for combinatorial chemistry are good. "A lot of drugs will be discovered with it," he says. "And it's hard to imagine that there will not be people who find some enormously interesting catalytic compounds and stoichiometric reagents using these methods." However, he says, "The real key to making it work is twofold. First, you really need to have a good idea - a good basic structure that has a real chance of doing something really interesting, and you want to manufacture that idea in as many variants as you can afford to screen."

A second and even greater challenge, says Still, is devising novel and effective assays. "You need assays for the property you want that can be run in parallel, so you can select the beads or the library members that you want just by simple inspection. You simply look at them and pull out the ones that have the right property."

Gordon agrees that greater assay development is needed. "The amount of molecular diversity and the number of molecules that are going to become available are going to dwarf the present screening

capacities," he says. "What's required is more individualized assays and assay miniaturization."

Miniaturization not only saves on the cost of proteins and other rare materials used in assays but also provides greater compatibility with the very small amounts of combinatorial compounds that are typically synthesized on beads. "Instead of 96-well microtiter dishes, which are the current standard in the pharmaceutical industry, you're going to see 1,000-well trays," Gordon predicts.

Ontogen's Moran believes that an increased focus on analytical chemistry is needed in the combinatorial field. "One needs to produce a reasonable amount of material in order to characterize any one compound that might be of interest in a library, either by mass spectroscopy or more preferably by proton NMR," says Moran. "The reason for this is that organic synthesis is not straightforward."

Different groups added to a core structure will affect the reactivity of library members to a differential extent, leading to possible failures of key synthetic steps. "So one needs to get a handle on how much of each component is being produced and whether you're actually making all the components in your library," he says. "Unless we have good analytical control over our experiments, it's going to be a challenge knowing what one's made." However, another researcher comments that, although it's important to be able to analyze hits, it's not practical or even desirable to analyze all library compounds.

Janda believes another key goal for the future is "to create a global library or universal library, where if you screen the library you'd find a hit for any type of target. It might not be a very potent hit, but you'd find a lead. People are trying to create a small library that would be very diverse that would give you leads to almost anything in the drug area. Some people call this combinatorial chemistry's Holy Grail."

However, Still says the universal library may prove to be as permanently elusive as the Holy Grail. In combinatorial chemistry, he says, "medicinal chemists ... design the first library of 1,000 to 100,000 compounds that has a good chance of acting on the target they are going after. Then they screen and make a new sublibrary based on the structure-activity relationships they find. I don't think any medicinal chemist would believe any single library of 10,000 compounds, no matter how carefully chosen, will contain leads for every medical target."

Schreiber suggests that combinatorial molecular recognition could become a fundamental tool for understanding protein function. One of the ultimate goals of the Human Genome Project is to discover the functions of human proteins, he says, and up to now this has been done with molecular biology techniques.

"Virtually all studies of the functions of proteins today involve making mutations in the genes that encode proteins and studying the effects," he explains. "This genetic approach to studying protein function is very powerful, but it is very slow and very inefficient. It's going to take centuries to study the function of all the proteins encoded by the human genome this way, and that's simply unacceptable."

In principle, this problem could be solved, he says, by using a "chemical genetics approach - where instead of making mutations in the gene encoding the protein you attack the protein itself by using organic ligands that bind to it." And such ligands can best be identified with combinatorial methods.

Hence, says Schreiber, "Chemical genetics could be the way in the future to solve the problem of protein function. There's a big advantage if you do it that way - because the very act of understanding protein function gives you a molecule that actually alters function. In terms of medical applications of the knowledge we seek, that's what one is ultimately trying to do."

Combinatorial chemistry, coupled to structural biology and cell biology, "is the most likely avenue to solve the protein binding problem," he says. "If we can combine those techniques, the consequences will be very exciting. It will lead to an era where biology is intimately coupled to chemistry, and where one might even say that chemistry, rather than genetics, will drive biology."

The ultimate usefulness of combinatorial chemistry for drug discovery and other applications remains to be proved. But Lilly's Kaldor - whose group developed by combinatorial means the CNS agent that has advanced to the clinic - is one researcher who is cautiously optimistic. "These techniques are more broadly applicable than crystal-structure-guided design methods because you don't have to have any knowledge of your receptor in order to apply them. ... You can develop a pharmacophore hypothesis much more quickly than you might have otherwise been able to do so. To date, we have used combinatorial chemistry for lead generation or lead optimization in over 50% of current Lilly projects and anticipate this percentage will increase with time."

Lilly's development of the CNS compound took less than two years from target identification to the beginning of clinical trials. This is "very fast," says Kaldor, "and we, of course, are being challenged by our management to repeat this success in every project we work on. ... It's a stunning example of what can be done if ... you apply combinatorial chemistry."

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Predictive Carcinogenicity: A Model for Aromatic Compounds, with Nitrogen-Containing Substituents, Based on Molecular Descriptors Using an Artificial Neural Network

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A back-propagation neural network to predict the carcinogenicity of aromatic nitrogen compounds was developed. The inputs were molecular descriptors of different types: electrostatic, topological, quantum-chemical, physicochemical, etc. For the output the index TD50 as introduced by Gold and colleagues was used, giving a continuous numerical parameter expressing carcinogenicity. From the tens of descriptors calculated, principal component analysis enabled us to restrict the number of parameters to be used for the artificial neural network (ANN). We used 104 molecules for the study. An $R_{cv}^2 = 0.69$ was obtained. After removal of 12 outliers, a new ANN gave an R_{cv}^2 of 0.82.

1. INTRODUCTION

Man is exposed to many chemicals of natural and synthetic origin. An urgent question concerns their potential negative effects on human health. To identify chemicals inducing toxicity and to limit the incidence of human cancers and other diseases, rodent bioassays are the principal methods used today. However, this approach is not altogether problem-free, on several accounts: (1) the cost of the assay (>1 million U.S. dollars per chemical); (2) the time needed for the tests (3−5 years); (3) ethical considerations and public pressure to reduce or eliminate the use of animals in research and testing;[1] (4) difficulties in the extrapolation to man.

We were interested in the prediction of carcinogenicity, but cancer is not a single disease. Several mechanisms are involved in the various processes leading to the different tumors. This makes the task of assessing the computational prediction particularly challenging. Dedicated expert systems have been employed for computerized prediction of carcinogenicity.[2,3] However, these have limitations.[1−3] These expert systems work mainly on the assumption that toxicity is linked to the presence of toxic residues, either defined by human experts or found by the expert system. In some cases, the expert systems also use some simple physicochemical parameters. A very recent book describes the state-of-the-art of the research in the prediction of toxicity.[4]

Another widespread approach for predicting toxicity relies on molecular descriptors, which refer to global properties or characteristics of the molecule. In recent years a huge increase in the number of studies of theoretical molecular descriptors has appeared in the literature, including their use in toxicity prediction.[5] In the case of expert systems chemical data can be handled in several formats, but with artificial neural network (ANN) molecular descriptors are more suitable, and indeed they have been used in the prediction of carcinogenicity with contrasting results.[6−8] In this study we consider the use of molecular descriptors as input to ANN for the prediction of carcinogenicity of aromatic compounds with nitrogen-containing substituents.

2. METHODS

2.1. Input and Output of the Model. In many cases the carcinogenicity of a compound is classified by activity. A numerical, continuous approach was introduced by Gold and colleagues.[9] Gold's database contains standardized results for carcinogenicity for more than 1200 chemicals; for each substance it reports the carcinogenicity on rat and mouse, expressed using the parameter TD50, which is the chronic dose rate that would give half the animals tumors within some standard experimental time—the "standard lifespan" for the species. The huge amount of information in the database and the quantitative homogeneous evaluation are two important advantages. This database was therefore adopted as the basis for selecting the output parameter for the neural network. In the present study, for each chemical we chose the lowest (i.e. most potent) TD50. For the purpose of homogeneity all data refer to the mouse.

We limited the chemical compounds to be evaluated to those containing an aromatic ring and a nitrogen linked to the aromatic ring, because our previous experience with a commercial expert system showed that several of the compounds classified incorrectly belonged to this category.[10] The category includes several chemical classes, such as nitrosamines, amides, amines, and nitro derivatives, etc. The list of 104 selected compounds, with their toxic activity, is given in Table 1.

For the output we transformed the TD50 as follows: output = log(MW × 1000/TD50) (MW = molecular

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Table 1. Chemical Names, CAS Number, and Experimental and Calculated Toxic Values of 104 Compounds

name	CAS no.	expt	pred	name	CAS no.	expt	pred
(*N*-6)-(methylnitroso)adenine		0.6665	0.4923	5-nitroacenaphthene	602-87-9	0.6194	0.6508
(*N*-6)-methyladenine	443-72-1	0.0000	0.4462	acetaminophen	103-90-2	0.0000	0.3215
1,5-naphthalenediamine	2243-62-1	0.5838	0.5713	AF-2	3688-53-7	0.5922	0.5664
1-(1-naphthyl)-2-thiourea	86-88-4	0.8274	0.6979	aniline·HCl	142-04-1	0.2679	0.2523
1-amino-2-methylanthraquinone	82-28-0	0.5516	0.6981	anthranilic acid	118-92-3	0.1737	0.1693
1-[(5-nitrofurfurylidene)amino]hydantoin	67-20-9	0.4588	0.4831	atrazine	1912-24-9	0.6881	0.6902
2,2′,5,5′-tetrachlorobenzidine	15721-02-5	0.5963	0.6738	azobenzene	103-33-3	0.7571	0.7360
2,2,2-trifluoro-*N*-[4-(5-nitro-2-furyl)-2-thiazolyl]acetamide	42011-48-3	0.7321	0.6992	benzidine·2HCl	531-85-1	0.7086	0.6738
				c.i. disperse yellow 3	2832-40-8	0.4769	0.4644
2,4,5-trimethylaniline	137-17-7	0.7129	0.6384	chloramben	133-90-4	0.3477	0.2602
2,4,6-trimethylaniline·HCl	6334-11-8	0.6498	0.6310	chlorambucil	305-03-3	1.0000	0.9094
2,4-diaminoanisole sulfate	39156-41-7	0.4965	0.4567	cinnamyl anthranilate	87-29-6	0.4017	0.4539
2,4-diaminotoluene·2HCl	636-23-7	0.5643	0.5146	d & c red no. 9	5160-02-1	0.4336	0.4384
2,4-dimethoxyaniline·HCl	54150-69-5	0.4257	0.4197	dacarbazine	4342-03-4	0.8653	0.5674
2,4-dinitrophenol	51-28-5	0.0000	−0.0145	dapsone	80-08-0	0.0000	0.4293
2,4-dinitrotoluene	121-14-2	0.0000	0.3873	fd & c red no. 4	4548-53-2	0.2512	0.2209
2,4-xylidine·HCl	21436-96-4	0.6608	0.5765	fd & c yellow no. 6	2783-94-0	0.2717	0.2126
2,5-xylidine·HCl	51786-53-9	0.4458	0.5227	fluometuron	2164-17-2	0.5344	0.4913
2,6-dichloro-*p*-phenylenediamine	609-20-1	0.4405	0.4430	formic acid 2-[4-(5-nitro-2-furyl)-2-thiazolyl]hydrazide	3570-75-0	0.7277	0.6196
2-(acetylamino)fluorene	53-96-3	0.7563	0.7638				
2-amino-4-(5-nitro-2-furyl)thiazole	38514-71-5	0.7243	0.6966	furosemide	54-31-9	0.4876	0.5560
2-amino-4-(*p*-nitrophenyl)thiazole	2104-09-8	0.7133	0.6690	hydrochlorothiazide	58-93-5	0.4514	0.5654
2-amino-4-nitrophenol	99-57-0	0.4384	0.4929	*m*-cresidine	102-50-1	0.5100	0.5057
2-amino-5-nitrophenol	121-88-0	0.3238	0.3026	*m*-phenylenediamine·2HCl	541-69-5	0.4844	0.4144
2-amino-5-nitrothiazole	121-66-4	0.0000	−0.0862	*m*-toluidine·HCl	638-03-9	0.3831	0.3642
2-aminoanthraquinone	117-79-3	0.4630	0.6501	melamine	108-78-1	0.3532	0.4286
2-aminodiphenylene oxide	3693-22-9	0.7344	0.7324	melphalan	148-82-3	0.9803	1.0032
2-biphenylamine·HCl	2185-92-4	0.4241	0.3075	methotrexate	59-05-2	0.6443	0.4927
2-chloro-*p*-phenylenediamine sulfate	61702-44-1	0.4001	0.4022	metronidazole	443-48-1	0.4927	0.4924
2-hydrazino-4-(5-nitro-2-furyl)thiazole	26049-68-3	0.6857	0.6391	mexacarbate	315-18-4	0.8264	0.8305
2-hydrazino-4-(*p*-aminophenyl)thiazole	26049-71-8	0.7018	0.6003	*N*-(1-naphthyl)ethylenediamine·2HCl	1465-25-4	0.0000	0.2226
2-hydrazino-4-(*p*-nitrophenyl)thiazole	26049-70-7	0.7134	0.6021	*N*-nitrosodiphenylamine	86-30-6	0.4952	0.4837
2-methyl-1-nitroanthraquinone	129-15-7	0.8404	0.7969	*N*-phenyl-*p*-phenylenediamine·HCl	2198-59-6	0.0000	0.4836
2-naphthylamine	91-59-8	0.6557	0.6456	*N*-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide	24554-26-5	0.7325	0.7051
2-nitro-*p*-phenylenediamine	5307-14-2	0.4532	0.2208				
2-*sec*-butyl-4,6-dinitrophenol	88-85-7	0.8360	0.8256	*N*-[5-(5-nitro-2-furyl)-1,3,4-thiadiazol-2-yl]acetamide	2578-75-8	0.7440	0.5990
3,3′-dimethoxybenzidine-4,4′-diisocyanate	91-93-0	0.2791	0.4109				
3-(3,4-dichlorophenyl)-1,1-dimethylurea	330-54-1	0.4788	0.6364	nithiazide	139-94-6	0.4609	0.4735
3-chloro-*p*-toluidine	95-74-9	0.3807	0.3849	nitrofen	1836-75-5	0.6198	0.5780
3-nitro-*p*-acetophenetide	1777-84-0	0.3995	0.4186	*o*-aminoazotoluene	97-56-3	0.5936	0.5913
4′-fluoro-4-aminodiphenyl	324-93-6	0.8306	0.5675	*o*-anisidine·HCl	134-29-2	0.4162	0.4160
4,4′-methylenebis(2-chloroaniline)·2HCl	64049-29-2	0.6141	0.6300	*o*-phenylenediamine·2HCl	615-28-1	0.4333	0.4379
4,4′-methylenebis(*N*,*N*-dimethyl)benzenamine	101-61-1	0.5456	0.5662	*o*-toluidine·HCl	636-21-5	0.4296	0.4531
4,4′-methylenedianiline·2HCl	13552-44-8	0.6760	0.6068	*p*-anisidine·HCl	20265-97-8	0.0000	0.3522
4,4′-oxydianiline	101-80-4	0.6680	0.5299	*p*-chloroaniline	106-47-8	0.3917	0.3951
4-amino-2-nitrophenol	119-34-6	0.0000	0.2578	*p*-cresidine	120-71-8	0.5986	0.5234
4-aminodiphenyl	92-67-1	0.8312	0.6604	*p*-isopropoxydiphenylamine	101-73-5	0.4703	0.4558
4-chloro-*m*-phenylenediamine	5131-60-2	0.4088	0.3924	*p*-nitrosodiphenylamine	156-10-5	0.5024	0.6000
4-chloro-*o*-phenylenediamine	95-83-0	0.4233	0.4286	*p*-phenylenediamine·2HCl	624-18-0	0.3813	0.3249
4-chloro-*o*-toluidine·HCl	3165-93-3	0.6942	0.6084	pentachloronitrobenzene	82-68-8	0.6161	0.6816
4-nitro-*o*-phenylenediamine	99-56-9	0.0000	0.3094	phenacetin	62-44-2	0.2859	0.3255
4-nitroanthranilic acid	619-17-0	0.2882	0.3812	phenylhydrazine	100-63-0	0.0000	0.0428
5-nitro-2-furaldehyde semicarbazone	59-87-0	0.6600	0.4923	proflavine·HCl hemihydrate	952-23-8	0.6535	0.6667
5-nitro-*o*-anisidine	99-59-2	0.4276	0.4530	pyrimethamine	58-14-0	0.5199	0.6243

weight), in order to have a more continuous output space and to refer to the moles of the chemical, not the weight.[8]

2.2. Molecular Descriptors. Chemical structures were drawn with Hyperchem (Hypercube, Inc.) and optimized using the PM3 Hamiltonian. We used the following programs to calculate descriptors: VAMP version 6.1 (Oxford Molecular Ltd.) for the quantum-mechanical and thermodynamic calculations, on a Silicon Graphics XS24 workstation; HAZARD EXPERT version 3.0 (CompuDrug Chemistry Ltd., Budapest, Hungary) for log *D* calculation; TSAR version 3.0 (Oxford Molecular) for the other descriptors, using a personal computer.

We calculated the 34 descriptors listed in Table 2. log *D* was calculated at pH 2, 7.4, and 10 as representative of the pH of the stomach, blood, and gut, where different processes may occur with the chemicals. The complete set of values is available from the authors on request.

Table 2. The 34 Used Descriptors

molecular weight	three principal axes of inertia
log *D* at pH 2, 7.4, 10	Balaban Index
HOMO	Wiener Index
LUMO	Randic Index
heat of formation	five Kier & Hall connectivity indices
dipole moment	
polarizability	six Kier shape indices
total energy	flexibility index
molecular volume	ellipsoidal volume
three principal moments of inertia	electrotopological sum

2.3. Reducing the Number of Descriptors by Principal Component Analysis. We used principal component analysis

(PCA) to select a smaller set of descriptors so the network could converge faster.

The main change in the set of 104 molecules (accounting for 63% of the total variability) was explained by the descriptor total energy and by a pool of descriptors including topological, geometric, and electrostatic values inversely correlated with the first principal component (PC). The second PC, accounting for another 8% of the variability, was mainly related to the dipole moment, the topological index of Balaban, and the quantum-chemical HOMO and LUMO descriptors and to log D at pH 7.4 and pH 10. The log D at pH 2 correlated with the third PC, thus explaining another smaller but different source of variability.

Descriptors with the highest scores on the first four components of PCA (accounting for 85% of the total variability) were chosen and reduced, eliminating those most closely correlated. A final criterion was to keep a pool of descriptors representing the different aspects of the molecule considered (physicochemical, electronic, and topological, etc). From the 34 descriptors calculated, 13 were selected: molecular weight, HOMO, LUMO, dipole moment, polarizability, Balaban, ChiV3 and flexibility indices, log D at pH 2 and pH 10, third principal axis of inertia, ellipsoidal volume, and electrotopological sum.

2.4. Artificial Neural Networks. In all the simulations, performed with MBP v 1.1,[11] the working parameters were set as follows: the weight initialized with the SCAWI technique; net gain $\eta(0) = 0.75$; initial moment $\alpha(0) = 0.9$; acceleration factor YPROP, $K_a = 0.7$, $K_d = 0.07$. The algorithm stopped itself when it encountered one of the following conditions: gradient lower than 10^{-6}; mean square error in validation (MSE) equal to 0; maximum calculated difference between calculated and desired output equal to 0; maximum number of iterations reached. Each network was trained starting from 100 random points in space, in order to minimize the probability of converging toward local minima. Input data were scaled between 0 and 1 in order to have a homogeneous range of variation of descriptors. The output was scaled accordingly.

For the validation step the leave-two-out approach was adopted, i.e. a cross-validation procedure using two examples in validation and the others for training. Five ANN models were generated, using data sets composed of 84 molecules randomly chosen in the training set and 20 in the test set.

The software is available on request, for noncommercial use.

3. RESULTS AND DISCUSSION

Most QSAR studies consider a limited number of parameters, taking account of previous knowledge in the field and using multivariate linear analysis. In our case there was no previous knowledge on the importance of specific molecular descriptors. We therefore considered a wide range of different classes, as detailed above, to extract information without a priori elimination of any possibilities.

We tried using regression analysis, but without success. ANN can be used to model complex phenomena where noise and nonlinear processes may be present, such as in our case. A disadvantage is the time needed, because many iterations are needed. This is a weakness of this neural network if we want to keep all the descriptors as inputs. Reducing the inputs shortens the training time. If this involves eliminating redundancy, the net has more chance of finding relevant parameters. For these reasons (reduction of computation time and elimination of redundancy) we chose PCA to select the ANN inputs, as it has been used for this purpose in other cases.[12,13] A risk related to the use of PCA is the possibility of eliminating inputs which behave nonlinearly. To verify that we had not eliminated any useful information, we built a new ANN using the first 12 PCs as inputs. These contain about 99% of the information of the original set of variables. The results with these PCs were comparable to those with the selected descriptors, shown below, indicating that we had not lost information through our selection.

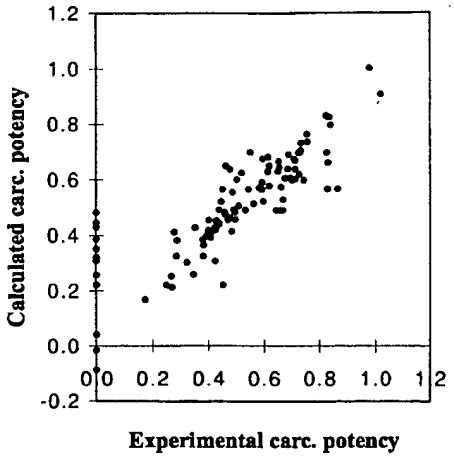

Figure 1. Predicted versus experimental carcinogenicity values with the BPNN four-neuron model.

Table 3. Results with BPNN of Increasing Numbers of Hidden Neurons (MSE = Mean Square Error)[a]

neurons	MSE	R_{cv}^2	neurons	MSE	R_{cv}^2
3	0.0157	0.675	6	0.0153	0.676
4	*0.0146*	*0.691*	7	*0.0146*	*0.691*
5	0.0154	0.676			

[a] Best results are in italics.

Table 1 gives results of the back-propagation neural network (BPNN). Figure 1 shows the predicted and experimental carcinogenicity with the BPNN four-neuron model.

The average R^2 cross-validated (R_{cv}^2) after 10 000 iterations and using different numbers of internal neurons is shown in Table 3.

To overcome possible representation bias in our data set, we built up five random data sets composed of 84 molecules in the training set and 20 in the test set. Then five independent ANN models were generated. This approach has been used recently.[14] R_{cv}^2 for these models was 0.70 using four or six neurons in the inner layer, in agreement with the leave-two-out method (see Table 3).

For the BPNN, the presence of outliers in the set was assumed and investigated in order to see whether the network's capacity for generalization improved after removing them and to assess the chemical nature of the activity of the compounds.

We adopted a conservative approach to remove outliers, taking only the molecules presenting an error in validation higher than 0.2 in the two best models (those with four and

Table 4. Results with BPNN of Increasing Number of Hidden Neurons, after Removing the Outliers (MSE = Mean Square Error)[a]

neurons	MSE	R_{cv}^2	neurons	MSE	R_{cv}^2
3	0.0062	0.793	6	0.0057	0.810
4	*0.0053*	*0.824*	7	0.0061	0.792
5	0.0053	0.824	8	0.0073	0.755

[a] Best results are in italics.

seven internal neurons). Twelve molecules were identified as outliers and removed. The results are presented in Table 4.

The results show that R_{cv}^2 has been clearly improved. Most of the outliers (9 out of 12) are molecules for which the experimental results for carcinogenicity were not statistically significant and an arbitrary value of 10^{31} was given in the Gold database (see Figure 1; they lie on the y axis, because of the transformation formula described in section 2.1 and scaling). The main experimental evidence for these molecules suggests noncarcinogenicity. Other considerations on the outliers regard their homogeneity from a chemical point of view. As we said, the compounds used for this ANN belong to several chemical classes and the outliers appear to be distributed over various chemical classes. Some are chemicals that have no structures in common with other members of the set, and this may explain their behavior. However, the ANN correctly predicted the toxicity of other chemicals which appear badly represented.

Special consideration must be given to two molecules, *o*- and *p*-anisidine. These isomers have identical or very similar chemical descriptors. However, their toxicity is very different, due to different metabolism in the animals. The ANN based on molecular descriptors was not able to distinguish them. This is a case of interesting behavior, shared with other compounds, which may undergo a metabolic process able to detoxify the chemical. In another study we solved the case of *o*- and *p*-anisidine by an expert system which distinguishes the toxic substructure.[15]

The present study illustrates the possibilities and limitations of the approach based on molecular descriptors. From the chemical point of view *o*- and *p*-anisidine may appear very similar, but for a living organism they are not. There are, however, chemicals which appear different within various chemical classes—as in the case of the compounds we have used—that the organism considers similar, because they are converted to aromatic amines. Knowledge of the body's bioprocesses is therefore an important source of information. Knowledge of the structural features of the molecule that characterize its specific mechanism of action cannot be ignored in some cases, in order to solve problems occurring in the prediction.

Another general point is the reliability of the database. We used an authoritative database, resulting from critical assessment of data from two sources: reports in the literature using different experimental protocols and results obtained according to a uniform protocol within the U.S. National Toxicology Program. Differences in the sources may affect the homogeneity of the data.[16] Furthermore, this database, like many others, changes constantly as new studies appear, adding knowledge.

A final comment on the database is that in most cases it still contains a limited number of compounds (despite the huge amount of work needed to build them up), so for some compounds we did not have enough examples to train the ANN properly.

4. CONCLUSIONS

Many models for toxicity prediction use linear relationships, which apply well within congeneric chemical classes. ANN has been used in limited cases. Villemin et al. used ANN to model polycyclic aromatic compounds in carcinogenic classes, obtaining good results.[6] Vracko obtained an r of 0.83, after removing the outliers, for a set of aromatic compounds belonging to different chemical classes.[8] Benigni and Richard, in a study using 280 compounds of various kinds, concluded that BPNN models fitted the training sets but had no general applicability.[7] The main feature of their study is the large differences between the structures of the molecules, much wider than in the other ANN used to predict carcinogenicity, including our present study.

The present study shows the feasibility of an ANN for predicting carcinogenicity of chemicals of various types. Several chemical classes are in fact present.

Our study attempts to illustrate how knowledge can be improved using ANN, probably because it is modeling nonlinearity. With chemical descriptors as input ANN is useful for cases where multilinear regression fails. We are aware of the limitations of this approach, which are common to other methods, as discussed. However, we believe that no single approach can cope with the vast problem of predictive toxicology, as already noted by other authors.[17] The next task is the extension to a wider set of chemicals. How to extract rules from the ANN is a major topic, and how to integrate ANN results with those from independent sources. We have already evaluated this last point in some cases, coupling expert systems and ANN within hybrid systems able to incorporate the best elements from each of the approaches.[15,18]

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